Proceedings of the Iowa Academy of Science

Volume 58 | Annual Issue Article 30

1951

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Journal Paper No. J-2058 of the Iowa Agricultural Experiment Station, Ames, Iowa. Project 1131.

Introduction

The existence of vitamin B_{12}, often designated the antipernicious anemia or animal protein factor, as another member of the expanding family of vitamins is well established. As implied from its nomenclature, it belongs to the water soluble B-vitamins, but unlike most other vitamins in this group its chemical structure is unknown.

Its nutritional importance to man, his monogastric farm animals and his poultry is recognized. For the present, at least, the supply consists primarily of concentrates. It is not to be inferred that crystalline B_{12} is unavailable. On the contrary, however, the supply is very limited and unitages of activity comparable to those in concentrates are much more costly. Consequently, the supply of crystalline material is limited largely to the pharmaceutical trade whereas the livestock and poultry industries rely principally upon concentrates.

To date, no dependable chemical procedure has been devised for quantitatively determining vitamin B_{12} in a variety of materials. Instead one must resort to bioassay procedures based on the growth stimulus of the vitamin to either bacteria (microbiological) or laboratory animals (macrobiological). These too have their limitations.

The determination of vitamin B_{12} by microbiological methods possesses certain advantages over those based on laboratory animals. It is obvious that these are mainly economy and rapidity of assay. However, in their present state of development these methods also possess definite inherent limitations. Whereas the assay data obtained by any specified microbiological procedure may be highly reproducable, the results may at the same time be at variance with those obtained by another microbiological method, Furthermore, uniform agreement with assays obtained from laboratory animals leaves much to be desired. Assay discrepancies often can be associated with the origin of the materials to be assayed, notably various products of animal origin. Also, these materials often require prior treatment thereby contributing futher to the possibility of divergent results.

It is a generally accepted fact that vitamin assays based on laboratory animals are both costly and time-consuming compared to microbiological procedures. Assays of vitamin B_{12} are no exception. However, animal assays do possess certain advantages. The physiological responses of laboratory animals to vitamin administration presumably are less readily influenced by casual alterations in environmental conditions than are those of bacteria. Furthermore, the physiological responses of laboratory animals to vitamin B_{12} should be more comparable to those exhibited by man and farm animals following their use of the same vitamin.

Our interest in vitamin B_{12} studies required the use of a reliable procedure for its determination in various materials such as body tissues and livestock feedstuffs of animal origin. Assays using labortaory animals appeared to be more reliable, and although admittedly very laborious to carry out, they are not necessarily prohibitive in cost. Several assay procedures which use animals have been reported (1, 2, 3, 4, 5, 6). It appeared after a study of the literature that either the method of Frost, et.al. (4) which employs a ration based on the animal protein casein or that of Register, et. al. (5) based on proteinaceous ingredients of vegetable origin would measure the B_{12} activity of materials of miscellaneous origin more dependably at present than microbiological assays.

The following results form the basis of a preliminary report in which the Frost and Register methods are compared for assessing vitamin B_{12} or B_{12}-like activity. The materials assayed were fresh kidney tissues obtained from groups of four different classes of marketable farm animals; namely, beef cattle, hogs, sheep and lambs. Attention will be called to certain procedural limitations and suggested modifications in methodology.

Procedure

The kidneys used in this study were obtained from the regular early summer run of lambs (wethers), mature sheep, hogs (barrows), and beef cattle (steers). The live animals had been shipped to Des Moines, Iowa, for commercial slaughter. Forty-eight kidneys were procured randomly during the same two-week period from each class of animals. Each kidney was representative of a single animal. All 192 (4 x 48) kidneys were frozen promptly following the slaughter of each animal and kept in this state preparatory to further processing.

Each kind of kidney was fed subsequently to suitably prepared test rats as a finely suspended emulsion pipetted daily into individ-

ual feeding cups. Listed chronologically the steps used in preparing each test material were as follows:

(a) the 48 frozen kidneys in each batch were chopped, finely ground in a powered mill and thoroughly mixed expeditiously while still very cold; (b) aliquants were sealed in glass jars and held at -15° F.; (c) periodically aliquants of each kind of kidney were defrosted in a refrigerator, diluted with an equal weight of distilled water, homogenized in a Waring Blendor until suitable for accurate delivery by pipette and (d) the diluted material was stored under refrigeration preparatory to feeding.

Vitamin B_{12} (Merck-Cobione) was used as the standard reference material. It was administered intraperitoneally on alternate days at levels which preliminary animal tests indicated should be reasonably satisfactory. These were 0.025, 0.050, and 0.100 micrograms of vitamin B_{12} per rat daily.

In these rat bioassays the Frost and Register procedures were adhered to rather rigidly, though minor modifications had to be instituted. These involved primarily a slight reduction in the number of rats used.

Weanling rats ranging in weight from 40 to 50 grams each were used in both procedures. They were restricted to "protamonized" basal rations during both a preliminary vitamin B_{12} depletion period and the subsequent experimental period. The Frost basal ration is based on purified casein and composed essentially of purified ingredients, whereas the comparable ration used in the Register method is based primarily on ingredients of vegetable origin, table I. The Frost and Register methods specify essentially seven-day and fourteen-day depletion periods, respectively. Both employ a two-week experimental period. Both require that the rats be caged separately in wire bottom cages and be fed their appropriate basal rations and distilled water ad libitum. In our tests the choice of each rat, the specific assay treatment it was to receive, and the location of the cage it was to occupy in the racks were determined randomly.

The determination of vitamin B_{12} activity is based on the total growth made simultaneously during the experimental period by comparative groups of rats receiving on the one hand specific amounts of the material to be evaluated, and on the other, graded allowances of pure vitamin B_{12} reference material. Total growth made per rat during the experiment may range from a few grams in the negative control groups to larger and larger amounts by those

Table I

Composition of basal rations used in the Frost and Register procedures for determining vitamin B_{12} activity

Ingredients (miscellaneous)	Amounts		Ingredients (vitamins)	Amounts per 100 gm. ration	
	Frost	Register		Frost	Register
	gm.	gm.		mg.	mg.
Casein (vitamin-test)	18		Riboflavin	3	0.3
Dextrin	69		Thiamin-HCl	3	0.3
*Salt mixture	4		Inositol	20	10.
Ruffex	2		Niacin	3	2.
Agar	1.5		Pyridoxine-HCl	5	0.2
Crisco	5		Biotin	0.01	0.01
Codliver oil	1	**	Folic acid	0.01	0.025
Sulfaguanidine	0.5		Ca pantothenate	5	2.
Yellow corn—ground		46.35	p-aminobenzoic acid	5	25.
Soybean oil meal		46.35	Menadione	5	–
Corn oil		5.	Choline-HCl	500.	100.
$CaHPO_4$		0.92			
$CaCO_3$		0.6			
NaCl (iodized)		0.44			
$MnSO_4 \cdot 4H_2O$		0.04			
Cystine	0.2	0.3			
"Protamone"	0.06	0.06			

*U.S.P. No. 2

**Fat soluble vitamins supplied as oleum percomorphum diluted 1:4 with corn oil. Two drops administered per os to each rat weekly.

receiving rations supplemented sub-optimally to optimally with vitamin B_{12}.

The results of the first comparative assays left much to be desired by way of agreement with each other, and possibly accuracy. Increasing the number of rats per group seemed to offer much promise. Therefore, in a subsequent test conducted essentially the same as described above, reference curves of response based on groups of 10 rats each instead of four or five were obtained. For want of sufficient equipment it was impossible to determine the B_{12} activity of the kidney tissues by using groups of 10 rats each instead of four or five in a manner similar to that described earlier. Use of the larger numbers of rats had to be limited to the accumulation of data for reference curves.

In addition arrangements were made with Dr. J. Kastellic of this station to conduct a microbiological assay of the same samples of kidney tissues for purposes of comparison, in as much as assays of this type (7, 8, 9, 10, 11, 12, 13) are used extensively for determining vitamin B_{12} or B_{12}-like activity. The organism used here was Lactobacillus leichmannii 313(9).

Results and Discussion

The growth obtained in rats by supplementing the basal rations of Frost and Register with one gram each of fresh beef, hog, sheep, and lamb kidney and that obtained simultaneously by intraperitoneal injections of graded doses of reference vitamin B_{12} is presented in table II. It is to be observed that notably better performance was obtained using the Register method. There was greater survival amongst the rats, the unsupplemented control group gained weight only moderately as was expected whereas the weight gained by the experimental groups administered increasing dosages of vitamin B_{12} was progressively greater and definitely more logarithimic than that attained by the Frost method. Apparently the seven-day depletion period prescribed by the Frost method compared with that of two-weeks by the Register procedure was insufficient to deplete this population of rats of their reserves of this vitamin.

An alternative step in procedure might have been to extend the depletion period. With the Frost type of basal ration the rate of mortality probably would have been increased thereby further vitiating the results.

The growth responses of the rats to gram samples of the various kidney tissues fell more completely within the critical region of the reference curve obtained by the Register procedure than for that of Frost. More accurate evaluation of the kidney potencies probably

Table II

Effect of vitamin B_{12} (Merck-Cobione) and the B_{12} or B_{12}-like activity of different kidney tissues on the growth of weanling rats

Supplement (amount per rat daily)	*Frost's method		(*) Register's method	
	Rats per group	Aver. total gain in wt. per rat	Rats per group	Aver. total gain in wt. per rat
	no.	gm.	no.	gm.
None	5	44.4±3.4	4	25.0±4.0
B_{12} (Cobione) 0.025 μg	**4	43.7±2.7	4	42.0±1.8
B_{12} (Cobione) 0.050 μg	**4	53.0±1.7	4	46.5±4.3
B_{12} (Cobione) 0.100 μg	5	54.0±3.1	4	61.3±5.8
Beef kidney 1 gm.	**3	50.3±5.9	4	54.3±3.1
Hog kidney 1 gm.	**3	55.0±2.3	4	59.3±4.3
Sheep kidney 1 gm.	5	53.4±4.8	4	61.5±4.9
Lamb kidney 1 gm.	5	63.4±3.9	4	64.8±2.9

*Depletion and experimental periods one and two weeks, respectively.
(*) Depletion and experimental periods two weeks each.
**One or two rats died during experiment, cause unknown.

Table III

Effect of intraperitoneal administration of vitamin B_{12} (Merck-Cobione) on the growth of weanling rats

Amount of vitamin B_{12} (Cobione) per rat daily	*Frost method		(*) Register method	
	Rats per group	Aver. total gain in wt. per rat	Rats per group	Aver. total gain in wt. per rat
	no.	gm.	no.	g.m
None	10	29.4±2.5	10	15.8±2.7
0.025 μg.	10	39.7±2.0	10	28.7±2.8
0.050 μg.	**9	47.2±2.5	10	41.2±2.3
0.100 μg.	10	47.8±1.5	10	51.1±2.9
0.200 μg.	10	49.3±1.6	**9	61.9±3.5

*Depletion and experimental periods one and two weeks, respectively.
(*) Depletion and experimental periods two weeks each.
**One rat died during experiment, cause unknown.

would have been obtained by both procedures had half-gram instead of gram quantities been fed.

In table III are presented the results obtained in the follow-up test conducted similarly, in which groups of 10 rats each instead of 4 or 5 were administered increasing dosages of the B_{12} reference material. In this test the experimental mortality was small in marked contrast to that which occurred in the previous test. The increases in weight gained by the groups which received supplemental injections of B_{12} again were more consistent in the Register method and increased throughout the range of levels of supplementation. In other words, the growth responses obtained were more logarithimic by the Register procedure. Consequently, the critical area of the resultant curve, figure I, extends a greater distance thereby permitting more latitude and accuracy in assessing the potency of unknowns. Furthermore, the groups of 10 rats each yielded data by both the Frost and Register procedures that described smoother and more typical biological curves. Also, the standard errors were uniformly less, tables II and III, than when the test groups consisted of smaller numbers of rats.

The results tabulated in tables II and III are presented graphically in figure I. Here the differences in utilitarian value between the Frost and Register methods and the advantages of the latter are portrayed more vividly. It is somewhat surprising, though gratifying in view of the difference in the number of rats comprising the groups, that both reference curves obtained by each method are essentially superimposable. Again, the critical assay range is greater in the reference curves plotted from data obtained by the Reg-

ister method. Had the rats used in the Frost method been depleted further of their B_{12} reserves, however, at the risk of increased mortality, the reference curves obtained by both assay procedures might have been more alike.

In view of the small number of rats used in the assay groups for kidney tissues, the comparative flatness of the Frost reference curve, and the large amount of vitamin B_{12} in the quantity of kidney tissues used, the results of the animal assays obtained by the Frost and

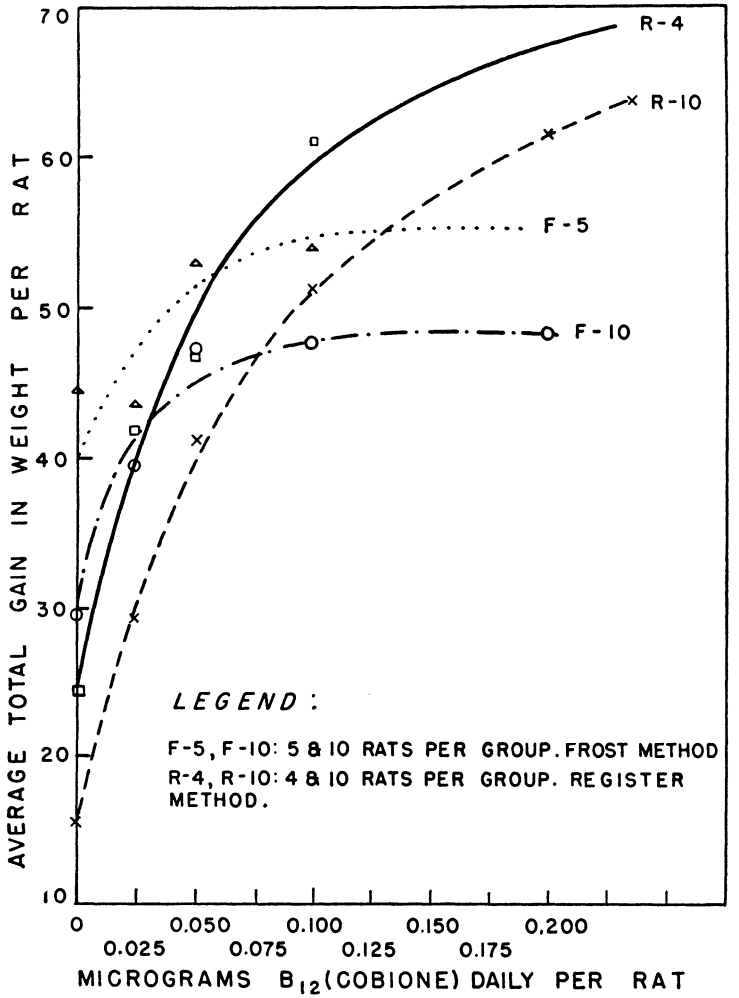

Fig.1 Frost & Register Reference Response Curves Used in Determining the Vitamin B_{12} Potencies of Kidney Tissues.

Table IV

Vitamin B_{12} or B_{12}-like activity of fresh kidney tissue determined by three bio-assay procedures

Source of kidney tissue	*Bio-assay method and B_{12} activity		
	Frost	Register	**Micro-biological
	μg./100 g.	μg./100 g.	μg./100 g.
Beef	4	6	10
Hogs	10	9	8
Sheep	7	11	21
Lambs	10	14	24

*These are essentially the potencies to be derived from the data. It is apparent that discrepancies exist between methods.

**Courtesy of Dr. J. Kastellic. Lactobacillus leichmannii 313.

Register methods are not markedly dissimilar, table IV. Also, included in the same table for comparison are the results of a microbiological assay of representative samples of the same kidney tissues. The microbiological assays of kidneys from cattle and hogs agree fairly well with those obtained using rats. On the contrary, the dissimilarity in results obtained for sheep and lamb kidneys by the micro- and macro-bioassay procedures is striking. Possibly the rat was unable to utilize efficiently all the B_{12} in sheep and lamb kidneys. A more plausable explanation is that these sheep and lamb kidneys may have contained something having vitamin B_{12}-like effects which enhanced the growth of the test organism used in this microbioassay; namely, *Lactobacillus leichmannii* 313.

Whatever may be the correct explanation the sum total of the results of this preliminary investigation indicate a need for further study of methodology in an effort to eliminate the factors responsible for these discrepancies. In the case of rat assays the use of sufficient animals to minimize experimental standard errors, and the use of amounts of the unknown which give assay responses that lie within the critical range of accurately determined reference curves are readily attainable prerequisites which would improve measureably the animal assay techniques. Also, the acceptability of the rat technique depends on the efficiency of this species in utilizing vitamin B_{12} whether it be ingested in naturally occuring materials or injected intraperitoneally as solutions of crystalline B_{12}.

The acceptability of microbiological techniques for assaying vitamin B_{12} in some materials is not questioned. However, it would seem presumptuous in the light of our present knowledge to assume that any of the micro-procedures now in use evaluate accurately the

vitamin B_{12} activity of all materials for which it might be used. Ultimately such an objective may be realized. In the meantime, it would appear highly desirable to frequently collect data simultaneously by microbiological and laboratory animal procedures for critical comparison.

Summary

The results of vitamin B_{12} bioassays of beef, hog, sheep, and lamb kidneys by the laboratory animal procedures of Frost, and Register, and the modified microbioassay procedure of Hoffman are not wholly in agreement. The implications of the discrepancies and some recommendations for their solution are discussed briefly.

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